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Research Paper

Host-dependent Induction of Transient Antibiotic Resistance: A Prelude to Treatment Failure

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ABSTRACT

Current antibiotic testing does not include the potential influence of host cell environment on microbial susceptibility and antibiotic resistance, hindering appropriate therapeutic intervention. We devised a strategy to identify the presence of host–pathogen interactions that alter antibiotic efficacy *in vivo*. Our findings revealed a bacterial mechanism that promotes antibiotic resistance *in vivo* at concentrations of drug that far exceed dosages determined by standardized antimicrobial testing. This mechanism has escaped prior detection because it is reversible and operates within a subset of host tissues and cells. Bacterial pathogens are thereby protected while their survival promotes the emergence of permanent drug resistance. This host-dependent mechanism of transient antibiotic resistance is applicable to multiple pathogens and has implications for the development of more effective antimicrobial therapies.

1. Introduction

Recent CDC estimates indicate that one in five pathogens from hospital-acquired infections in the U.S. are multidrug-resistant (Kallen et al., 2010; Sievert et al., 2013), dramatically limiting therapeutic options to antibiotics that may be more toxic, less effective, or more expensive (Centers for Disease Control and Prevention, 2013). In such cases, patients often have longer hospital stays, delayed recuperation, long-term disability, and increased mortality. Deciphering the mechanisms that govern the emergence of multidrug-resistant pathogens is critical to the development of new approaches to control bacterial infections. Many mechanisms of antibiotic resistance have been established, including horizontal gene transfer; genomic mutation; and intrinsic bacterial mechanisms that pre-date antibiotics (Allen et al., 2010; Andersson and Hughes, 2010; Cox and Wright, 2013; D'Costa et al., 2011; Davies and Davies, 2010). Significant advances have been made regarding the generation of antibiotic resistant variants (phenotypic and genotypic) that emerge during infection; *e.g.*, *Staphylococcus aureus* small colony variants that promote persistent infections (Proctor et al., 2006); antibiotic resistance of *Pseudomonas aeruginosa* biofilms (Høiby et al., 2010); the evolution and spread of multidrug-resistant pneumococcal variants (Croucher et al., 2011), and heteroresistant subpopulations of vancomycin-susceptible *S. aureus* (El-Halfawy and Valvano, 2015). Despite this knowledge, the role of host–pathogen interactions in antibiotic resistance is poorly understood, and the use of host models as a primary approach to understanding resistance is not often considered or explored.

For the past several decades, drug development has followed a standard sequential procedure wherein: (i) efficacy is determined *in vitro*; (ii) pharmacokinetic/pharmacodynamic (PK/PD) parameters are measured *in vivo*; and (iii) dosing efficacy/toxicity *in vivo* is established for a limited number of model pathogens (Ambrose et al., 2007; Clinical and Laboratory Standards Institute, 2012; Food and Drug Administration, 2009). However, along with a limited amount of patient-dosing data, physicians rely on *in vitro* antimicrobial susceptibility testing (AST) of clinical isolates grown on the universal media standard Mueller–Hinton Broth (MHB) for therapeutic intervention (Clinical and Laboratory Standards Institute, 2012; European Committee on Antibiotic Suscepibility Testing, 2014). This standard procedure does not replicate mammalian biochemistry and may not correlate with patient outcome. To overcome these limitations, we investigated antibiotic resistance in the context of animal models of disease, and have identified a mechanism

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that stimulates bacterial resistance to multiple antibiotics during infection, while promoting the emergence of drug-resistant bacteria.

2. Materials and Methods

2.1. Bacterial Strains and Media

TIVAR+ *S.* Typhimurium ATCC 14028 (CDC 6516–60) and MT2057 (an isogenic KAN resistant derivative of 14028), or TIVAR− *S.* Typhimurium var. 5 (04)-9639 (a multidrug-resistant isolate) derived from chicken and cow, respectively, were used in these studies (Conner et al., 1998; Heithoff et al., 2008). These strains have identical oral and i.p. lethal dose 50s (LD_{50}) in BALB/c mice, 10^5 and <10 colony forming units (CFU), respectively (Heithoff et al., 2008). *Salmonella* human clinical isolates were obtained from fecal and blood samples derived from patients with gastroenteritis or bacteremia, respectively; animal isolates were derived from different disease outbreaks, individual cases, or surveillance submissions to diagnostic laboratories (Heithoff et al., 2008). Unless otherwise specified, *Salmonella* were derived from stationary phase cultures aerated at 37 °C containing the Mueller–Hinton broth (MHB) (Clinical and Laboratory Standards Institute, 2012); low phosphate, low magnesium medium (LPM) (Coombes et al., 2004) or N-minimal medium (Nelson and Kennedy, 1971) supplemented with 0.3% glycerol and 0.1% casamino acids; or the Luria–Bertani (LB) medium (Davis et al., 1980). *Yersinia pseudotuberculosis* IP32953 is a virulent human isolate (Chain et al., 2004), and was assayed from stationary phase cultures aerated at 28 °C containing MHB or LPM media.

2.2. MIC Assays

The minimum inhibitory concentration (MIC) was determined in MHB and LPM media (pH 7 and pH 5.5) according to Clinical and Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute, 2012; Wiegand et al., 2008). MIC assays were performed on bacteria obtained from overnight culture, and from bacteria derived from macrophage lysates or host tissues. Bacteria were diluted in 0.15 M NaCl, and a 5 μl volume containing 10^4 CFU was spotted on agar plates of the media condition indicated, containing two-fold dilutions of each antibiotic. MIC values were derived following 20 h incubation at 37 °C (*Salmonella*) or 48 h incubation at 28 °C (*Y. pseudotuberculosis*), and were the result of at least three independent determinations.

2.3. Bacterial Cell Survival Assays

Bacterial cell survival was evaluated after cells derived from a given growth condition were exposed to antibiotics under the same or different growth condition (Groisman et al., 1997). *S.* Typhimurium 14028 was grown overnight in non-inducing medium for the TIVAR phenotype (N-minimal medium with 10 mM Mg^{2+} pH 7.7), diluted 1:100 in either inducing medium (N-minimal medium with 10 μM Mg^{2+} pH 5.8) or non-inducing medium (N-minimal medium with 10 mM Mg^{2+} pH 7.7), and incubated 4 h at 37 °C. Bacteria were diluted 1:200 and exposed to polymyxin B for 1 h in either inducing medium (N-minimal medium with 10 μM Mg^{2+} pH 5.8) or non-inducing medium (LB), and plated for CFU on LB medium. Percent survival was calculated as $CFU_{[polymyxin\ B]} / CFU_{[no\ drug]} \times 100$ at 1 h post drug exposure; values given are the REML model means ± SEM derived from at least 5 independent determinations.

2.4. Virulence Assays

Intraperitoneal (i.p.) infection. Salmonella grown overnight in LB medium were resuspended in 0.15 M NaCl and administered to mice via the i.p. route of infection (dose 10^2 or 10^3 CFU) (Heithoff et al., 1999). Five days post-infection, the bacterial cells were recovered from the spleen and other tissues/fluids of acutely infected animals. *Antibiotic treatment.* Mice infected i.p. with *Salmonella* (i.p. dose 10^2 CFU) were treated (or mock-treated) with a twice-daily polymyxin B or ciprofloxacin dosing regimen (30 mg/kg/day). Bacterial cells were recovered from the spleen of acutely infected animals and enumerated by direct colony count. Mouse survival was assessed for 10 days post-infection. Uninfected mice were also treated with polymyxin B to control for dosing toxicity. Six- to twelve-week old female BALB/c mice were used in all virulence studies. Institutional Animal Care and Use Committee of the University of California, Santa Barbara approved all mouse research protocols undertaken herein.

2.5. Cell Culture

The murine macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection, Rockville, MD., and maintained in minimum essential medium (MEM) supplemented with L-glutamine and 10% heat-inactivated bovine growth-supplemented calf serum (HyClone Laboratories, Logan, UT). Cells were grown in a humidified atmosphere of 5% carbon dioxide and 95% air at 37 °C in 75-cm^2 plastic flasks (Corning Glass Works, Corning, NY). Cultured murine macrophages (RAW 264.7) were harvested by scraping with a rubber policeman and plated at a density of 2.5 to 5×10^5 cells/ml in 4 ml of culture medium in 35 mm-diameter, six-well dishes (Corning) and grown for 24 h to approximately 80 to 90% confluence (1 to 5×10^6 cells/well) (adapted from previous methods (Fleckenstein et al., 1996)).

2.6. Bacterial Infection of Cultured Murine Macrophages

Bacterial cells were used to infect cultured murine macrophage (RAW 264.7) monolayers grown in cell culture plates (Corning) at a multiplicity of infection (MOI) of 10:1. The bacteria were centrifuged onto cultured monolayers at 1000 ×*g* for 10 min at room temperature, after which they were incubated for 30 min at 37 °C in a 5% CO_2 incubator. The co-culture was washed once with cell culture medium and incubated for 45 min in the presence of gentamicin (100 μg/ml) to kill extracellular bacteria, washed once with pre-warmed cell culture medium, and incubated in 4 ml of culture medium containing polymyxin B at the concentration indicated or 10 μg/ml gentamicin ($t = 0$ time point) for 24 h (adapted from previous methods (Finlay and Falkow, 1988)).

2.7. Statistical Analyses

Log transformed intracellular CFU and PMB^r mutation frequency data were analyzed using ANOVA (ANOVA, GenStat, 15th Edition, VSN International, UK). Intracellular CFU and PMB^r mutation frequency data are presented as the means ± standard error of the mean (SEM). Cell survival was analyzed using residual (or restricted) maximum likelihood (REML) analysis (GenStat, 15th Edition, VSN International, UK). A single variate model was used to analyze percentage survival on a log scale. The fixed effects of the model were the factors group, drug concentration, and their interaction. The Wald chi-square test was used to determine significant main effects and/or significant interactions between factors. Any non-significant terms were dropped from the model and analysis repeated. Following analysis, data are presented as predicted model-based means, *i.e.*, predicted means are those obtained from the fitted model rather than the raw sample means. Differences between the individual means calculated using ANOVA and REML were determined by calculating an approximate least significant difference (LSD). A difference of means that exceeded the calculated LSD was considered significant. Statistical significance for difference in proportions of animal survival was calculated using Chi-square (Epi Info 7, CDC). For all statistical analyses, a significance level (P) of less than 0.05 was considered to be statistically significant. Degrees of statistical significance are presented as $^{***}P < 0.001$, $^{**}P < 0.01$, or $^{*}P < 0.05$.

3. Results

3.1. Bacteria Exhibit High-level Antibiotic Resistance under Conditions That Simulate Host Microenvironments

The biochemical environment within certain host sites may induce transient changes in the bacterium that confer resistance to antibiotics. Thus, we screened a collection of pathogenic strains for antibiotic resistance under experimental conditions that simulated host microenvironments, including those present in the macrophage phagosome, a subcellular organelle in which *Salmonella* resides and replicates (Steele-Mortimer, 2008). Conditions within the phagosome can be mimicked by mildly acidic culture medium (pH 5.5) that is low in phosphate and magnesium (LPM) (Coombes et al., 2004). We compared the efficacy of 16 clinically-relevant antibiotics against *Salmonella* grown in LPM pH 5.5 medium versus that grown in MHB (unbuffered ~ pH 7.2) via assessment of the minimum inhibitory concentration (MIC) of each antibiotic (Clinical and Laboratory Standards Institute, 2012; European Committee on Antibiotic Susceptibility Testing, 2014). To control for the potential effects of pH and media composition, antibiotic resistance was determined by comparing the MICs in LPM medium versus those obtained in MHB at pH 5.5 and pH 7.

Growth of *S. enterica* Typhimurium in LPM pH 5.5 medium was linked to high-level resistance to polymyxin B (PMB; 64-fold) and colistin (CST; 256-fold), cationic peptides that disrupt Gram-negative membranes (Bergen et al., 2012; Landman et al., 2008) (Fig. 1A). We also observed a mild resistance to trimethoprim (TMP; 4-fold), an inhibitor of folate metabolism (Burchall, 1973). Under these conditions, *Salmonella* remains viable, and continues to grow at antibiotic concentrations that far exceed those achieved in treating human infections (0.5 to 2.5 mg/l) (Michalopoulos and Falagas, 2011; National Institutes of Health, 2014b; Sandri et al., 2013; Zavascki et al., 2008). Some other antibiotics examined were subject to pH and/or media composition effects on drug efficacy including kanamycin, streptomycin, and ciprofloxacin. In those cases, we could not determine the induction of antibiotic resistance. In other cases the efficacy of antibiotics was altogether unaffected, including tetracycline, chloramphenicol, and ceftiofur. Many pathogenic *Salmonella* serovars (serotypic variants) derived from human and livestock infections (Heithoff et al., 2008) exhibited high-level resistance to antibiotics PMB, CST and TMP when grown in LPM pH 5.5 medium: *S.* Typhimurium (5/6), *S.* Enteritidis (1/1), *S.* Dublin (2/2), *S.* Newport (1/2), *S.* Bovismorbificans (1/2), and *Salmonella* C1 K00-670 (1/1) (Supplementary Table 1). These data indicate that induction of antibiotic resistance under conditions simulating the phagosome is not a strain-specific phenomenon.

To ascertain whether the induction of antibiotic resistance exists in other microbial species, we screened for this phenotype in *Y. pseudotuberculosis*, a Gram-negative extracellular pathogen that

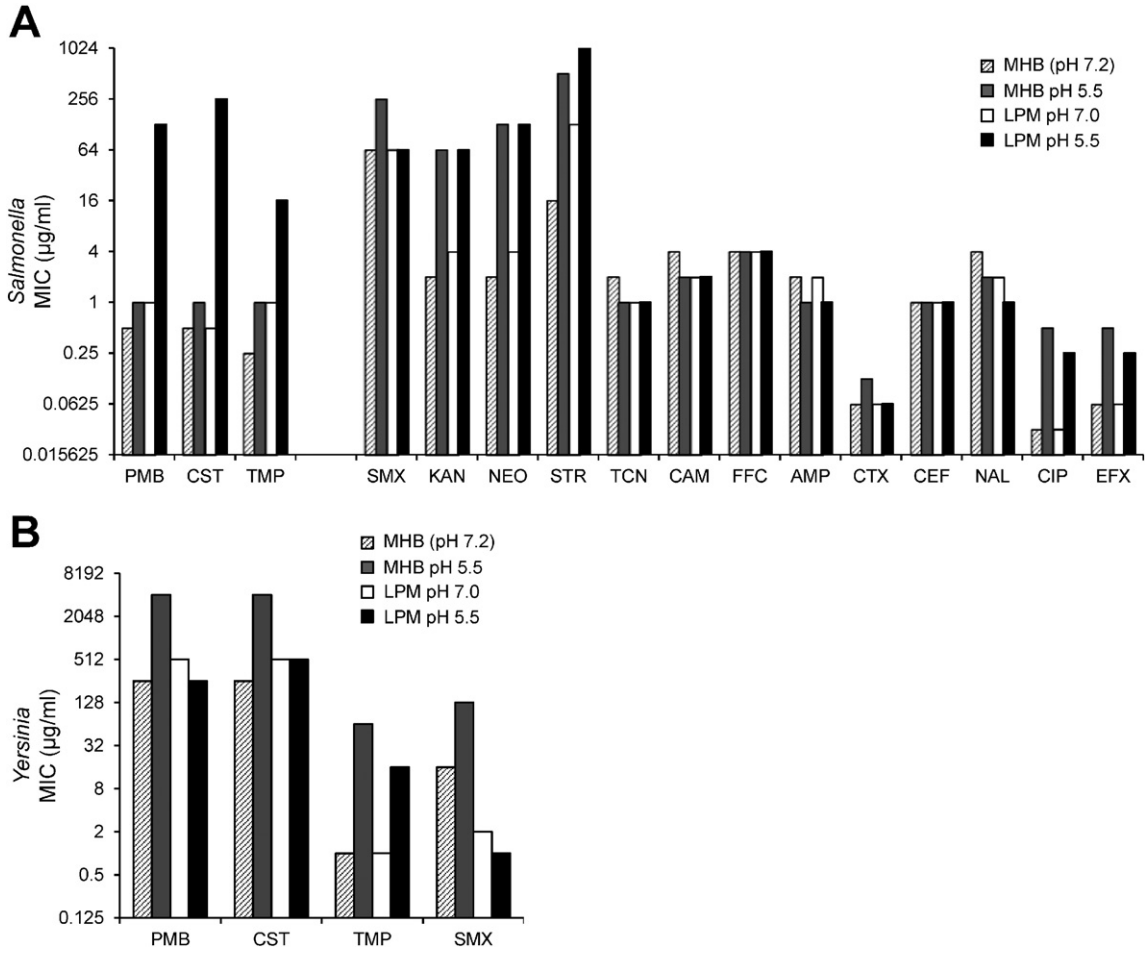

Fig. 1. *Salmonella* and *Yersinia* exhibit high-level antibiotic resistance under conditions that simulate host microenvironments. (A) *S.* Typhimurium 14028 was grown in either the Mueller–Hinton Broth (MHB) (Clinical and Laboratory Standards Institute, 2012) or low phosphate, low magnesium medium (LPM) (Coombes et al., 2004) at the pH indicated, and the minimum inhibitory concentration (MIC) of a panel of antibiotics was determined in the same medium (Clinical and Laboratory Standards Institute, 2012; Wiegand et al., 2008). The effect of growth conditions on antibiotic resistance was calculated by comparing the MIC in LPM medium divided by the MIC in MHB medium at both pH 5.5 and pH 7 (unbuffered) (ratio of LPM pH 5.5/pH 7.0 to MHB pH 5.5/pH 7.2). Drugs: polymyxin B, PMB; colistin sulfate, CST; trimethoprim, TMP; sulfamethoxazole, SMX; kanamycin, KAN; neomycin, NEO; streptomycin, STR; tetracycline, TCN; chloramphenicol, CAM; florfenicol, FFC; ampicillin, AMP; ceftriaxone, CTX; ceftiofur, CEF; nalidixic acid, NAL; ciprofloxacin, CIP; enrofloxacin, EFX. (B) The degree of *Y. pseudotuberculosis* IP32953 susceptibility to antibiotics as a function of growth conditions. MIC values were obtained from at least 3 independent determinations.

can cause severe disease in humans and livestock (Galindo et al., 2011; Tauxe, 2013). After normalizing for pH and media composition effects on drug efficacy, *Y. pseudotuberculosis* acquired increased resistance not only to antibiotics PMB, CST, and TMP (32-fold, 16-fold, and 4-fold, respectively), but also to sulfamethoxazole (SMX) (16-fold), another inhibitor of folate metabolism (National Institutes of Health, 2014a) (Fig. 1B; Supplementary Table 2). Further, the induction of *Yersinia* antibiotic resistance occurred under markedly different environmental conditions compared to *Salmonella* (in MHB pH 5.5 but not in LPM pH 5.5), perhaps reflecting the dissimilar host intracellular and extracellular trafficking of these bacterial species (*Salmonella* exposure to defensins within the phagosome (Steele-Mortimer, 2008); *Yersinia* exposure to cryptdins in the small intestine (Bevins and Salzman, 2011)). Together, these findings suggest that the scope of resistance induction may include a variety of different species; and current methods used to assess the degree and spectrum of antibiotic resistance do not account for environmental influences on microbial susceptibility *in vivo*.

3.2. Phenotypic Switching to High-level Antibiotic Resistance Is Rapid and Rapidly Reversible

To understand the mechanistic nature of phenotypic switching between antibiotic susceptible to resistant states, the kinetics and degree of antibiotic resistance induction was evaluated upon transfer of bacterial cells from MHB (non-inducing) to LPM pH 5.5 (inducing) medium. For this analysis, comparisons were made between a natural *Salmonella* isolate that was capable of inducing high-level resistance (*S.* Typhimurium 14028), with one that was not (*S.* Typhimurium var. 5 (04)-9639) (Supplementary Table 1). Transfer of *S.* Typhimurium 14028 from MHB to LPM pH 5.5 medium resulted in a rapid MIC change from a PMB susceptible to resistant phenotype within 1 cell doubling (Fig. 2A). Upon subsequent transfer from LPM pH 5.5 back to MHB medium, bacteria reverted to the susceptible phenotype within 4 to 5 generations. In contrast, transfer of *S.* Typhimurium var. 5 (04)-9639 from MHB to LPM pH 5.5 medium did not result in high-level PMB resistance. These data indicate that, for isolates capable of resistance induction, phenotypic switching to high-level antibiotic resistance is rapid and rapidly reversible. We then examined whether resistance induction can occur in the absence of rapid cell division and mutational selection via transferring bacterial cells from an overnight MHB culture into LPM pH 5.5 medium. It is anticipated that such a media shift markedly slows bacterial cell division since the final cell density of a saturated culture in rich MHB medium is considerably greater (~5-fold) than that in LPM pH 5.5 medium (Heithoff et al., 2012). Transfer of *S.* Typhimurium 14028 from MHB to LPM pH 5.5 medium, without dilution, resulted in a rapid change in MIC from a PMB susceptible to resistant phenotype after a short incubation period (Fig. 2B). These findings indicate that

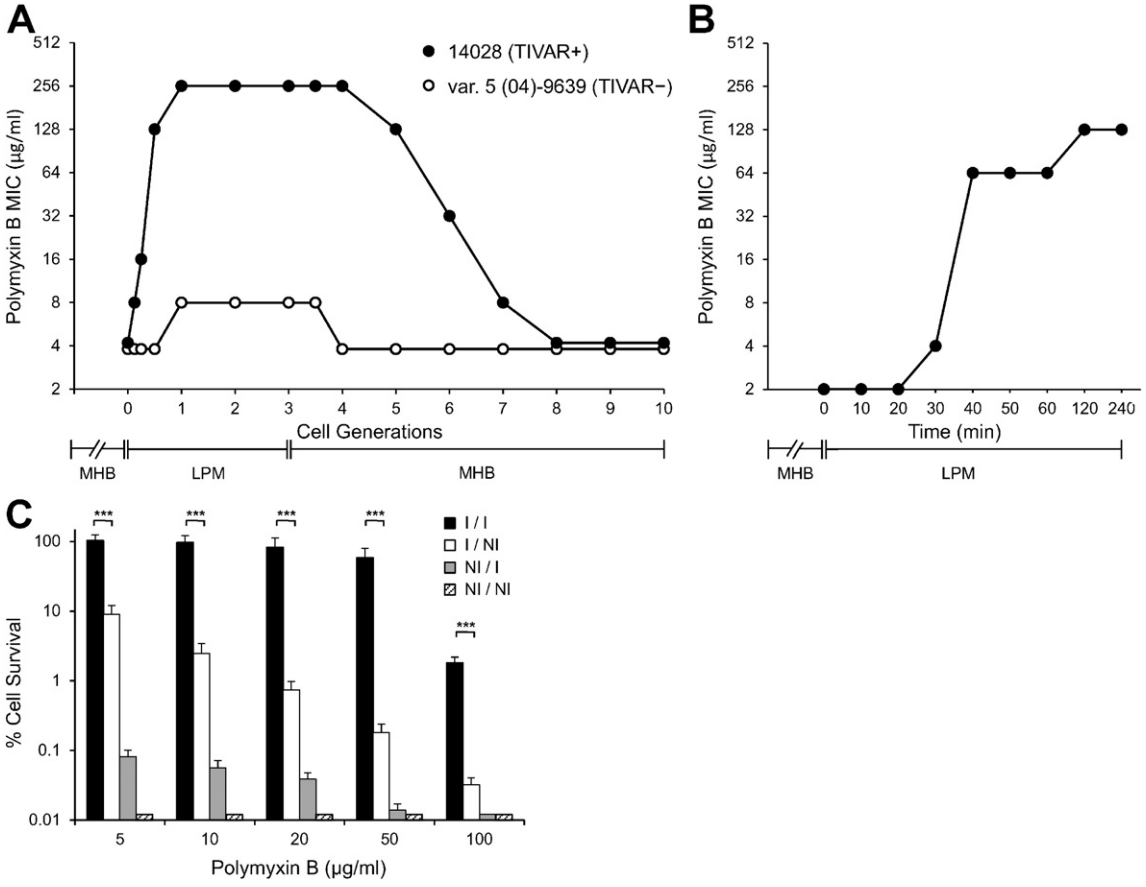

Fig. 2. Induction of high-level antibiotic resistance in *Salmonella* is rapid and rapidly reversible. (A) TIVAR+ *S.* Typhimurium 14028 and TIVAR− *S.* Typhimurium var. 5 (04)-9639 were grown for 4 cell generations (cell doublings) in MHB (log phase) and transferred to LPM pH 5.5 medium for 3 cell generations; subsequently these cells were transferred back into MHB medium for 7 cell generations (Heithoff et al., 2012). (B) *S.* Typhimurium 14028 was grown overnight in MHB medium, and bacterial cells from the saturated culture were transferred without dilution to LPM pH 5.5 medium. MIC values were determined in LPM pH 5.5 medium containing polymyxin B from at least three independent determinations. (C) The effect of growth conditions prior to and during drug exposure on *Salmonella* susceptibility to polymyxin B in bacterial cell survival assays (Groisman et al., 1997). *S.* Typhimurium 14028 grown (4 h) under inducing (*I*) or non-inducing (*NI*) conditions for TIVAR were subsequently exposed to PMB (1 h) under *I* or *NI* conditions, with CFU determined on nonselective LB medium. Black bars (*I*/*I*), growth and drug exposure (N-minimal medium with 10 μM Mg^{2+} pH 5.8). White bars (*I*/*NI*), growth (N-minimal medium with 10 μM Mg^{2+} pH 5.8); drug exposure (LB). Gray bars (*NI*/*I*), growth (N-minimal medium with 10 mM Mg^{2+} pH 7.7); drug exposure (N-minimal medium with 10 μM Mg^{2+} pH 5.8). Hatched bars (*NI*/*NI*), growth (N-minimal medium with 10 mM Mg^{2+} pH 7.7); drug exposure (LB). Percent survival = $CFU_{[polymyxin\ B]}$ / $CFU_{[no\ drug]}$ × 100 at 1 h post drug exposure. Statistical significance for cell survival was analyzed using REML analysis. Values given are the REML model means ± SEM derived from at least 5 independent determinations. ***$P < 0.001$; limit of detection < 0.02%.

phenotypic switching between antibiotic susceptibility to resistance does not require rapid cell division and mutational selection. Since this resistance mechanism is rapidly reversible and induced under experimental conditions that simulate host microenvironments, we have termed this mechanism TIVAR, transient *in vivo* antibiotic resistance.

Next, the number of resistant cells in the bacterial population was evaluated as a function of environmental conditions in bacterial cell survival assays, wherein cell viability is monitored after drug treatment (Groisman et al., 1997; Gunn and Miller, 1996). Our findings indicate that more than 80% of the bacterial population survived a high-dose PMB treatment (20 μg/ml) only if both prior growth and drug exposure occurred in inducing (*I*/*I*) conditions (Fig. 2C); cell survival was markedly decreased if either transpired in non-inducing conditions (*I*/*NI*; *NI*/*I*; *NI*/*NI*). Together, these data indicate that TIVAR induction involves a substantial portion of the bacterial population and requires both prior cell and drug exposure under inducing conditions.

3.3. The role of the environment in microbial susceptibility to antibiotics

Since the PhoPQ/PmrAB regulons contribute to resistance to cationic peptides via LPS remodeling of Gram-negative membranes (Chen and Groisman, 2013; Gunn, 2008), we tested whether these regulatory functions were required for the TIVAR phenotype. Introduction of *pmrA* and/or *phoP* null mutations into TIVAR+ *S.* Typhimurium abrogated PMB resistance under inducing and non-inducing conditions; whereas introduction of constitutively active mutations in *pmrA* or *phoQ* (Groisman et al., 1997; Gunn and Miller, 1996; Roland et al., 1993; Tamayo et al., 2005) resulted in high-level PMB resistance under non-inducing conditions (Fig. 3A). None of these mutations affected the induction of trimethoprim or ciprofloxacin resistance, suggesting that the host microenvironment may influence microbial susceptibility through multiple regulatory pathways, each operating in antibiotic-specific fashion.

The PmrAB regulatory system promotes resistance to toxic metals and cationic peptides in response to *ex vivo* signals (*e.g.*, high Fe^{3+}), which activate PmrB and downstream genes involved in Gram-negative membrane remodeling (Wösten et al., 2000). Thus, we reasoned that some naturally occurring TIVAR− isolates could potentially be converted to TIVAR+ under high Fe^{3+} conditions via stimulation of the PmrAB regulon and resultant bypassing of inherent mutational deficiencies in membrane stabilization. To test this hypothesis, we screened a collection of *Salmonella* clinical isolates for susceptibility to PMB under LPM pH 5.5 conditions. These studies revealed that TIVAR− isolates represent a significant subset of natural *Salmonella* populations derived from human and livestock infections (Heithoff et al., 2008): *S.* Typhimurium (10/61), *S.* Newport (2/10), *S.* Enteritidis (2/8), *S.* Dublin (0/8), *S.* Bovismorbificans (2/5), and *S.* Choleraesuis (3/3). Subsequently, we assessed whether TIVAR− isolates could convert to TIVAR+ under high Fe^{3+} conditions. Growth of TIVAR− *S.* Typhimurium strains in LPM pH 5.5 medium in the presence of 100 μM $FeSO_4$ resulted in high-level PMB resistance in 5 of 7 strains tested; 2 of 7 strains exhibited no growth in the presence of 100 μM

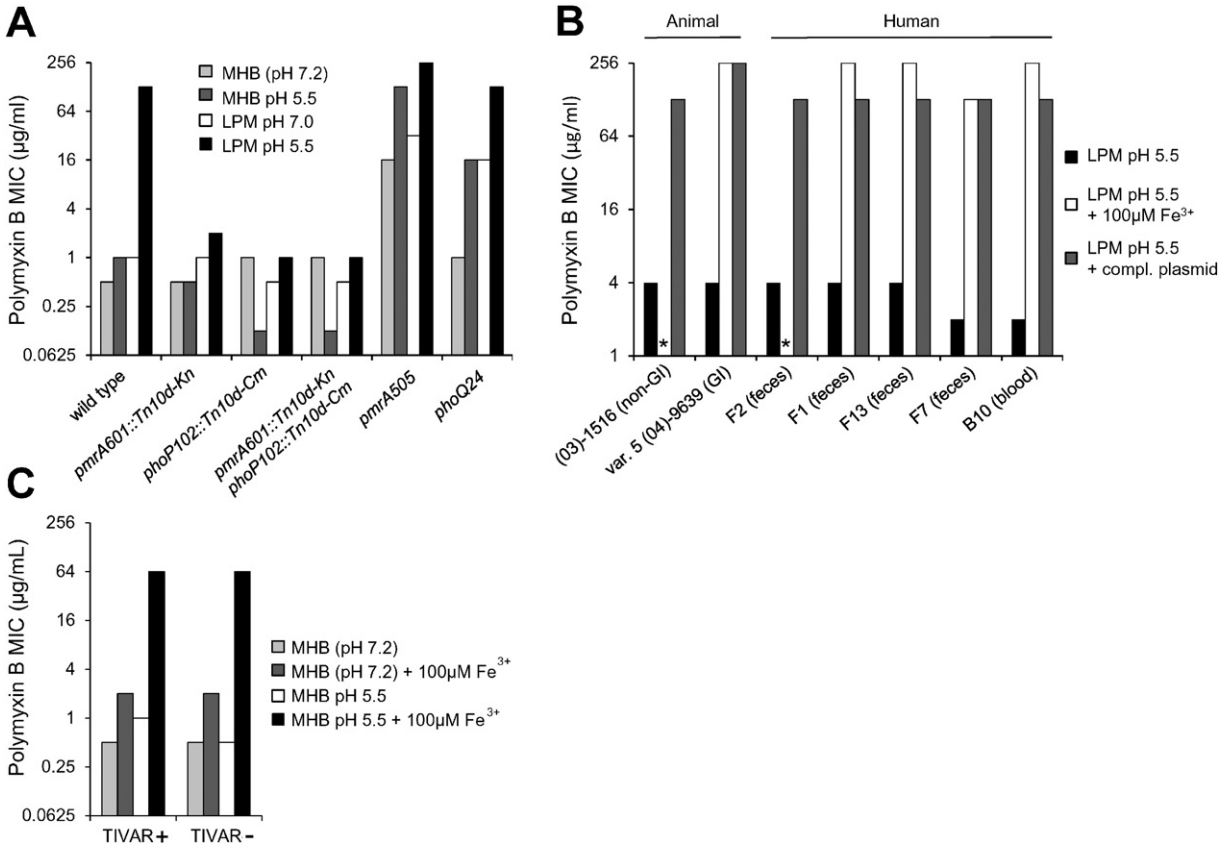

Fig. 3. The role of the environment (Fe^{3+}) in microbial susceptibility to antibiotics. (A) *pmrAB* and *phoPQ* null (*pmrA601*::Tn*10d*-Kn, *phoP102*::Tn*10d-Cm*) and constitutively active mutations (*pmrA505*, *phoQ24*) were introduced into *S.* Typhimurium 14028. Bacteria were grown in either LPM or MHB at pH 5.5 or pH 7 and MIC values were determined in the same medium from at least three independent determinations. (B) Bacteria were grown in LPM pH 5.5 medium in the presence and absence of 100 μM $FeSO_4$, or in the presence of a complementing plasmid containing wild-type sequences of the indicated mutation. * denotes no growth in the presence of 100 μM $FeSO_4$ due to iron toxicity. GI; gastrointestinal. (03)-1516 (horse non-GI) $pmrAB_{P1}$ -72C>T, *pmrB* H152Y; F2 (human feces) $pmrAB_{P1}$ -72C>T, *pmrB* H152Y; F1 (human feces) $pmrAB_{P2}$ 1599G>A; F13 (human feces) $pmrAB_{P2}$ 1408T>C, *pmrC* E415Q; F7 (human feces) *phoQ* P83L. Both B10 (human blood) and var. 5 (04)-9639 (cow GI) strains were complemented to TIVAR+ with recombinant PmrD+ sequences (Roland et al., 1994); corresponding mutations were not found in *pmrD*, *phoPQ*, or *pmrCAB*, suggesting parental mutation(s) are within other genes in the PhoPQ/PmrAB pathway. (C) Bacteria were grown in MHB pH 7.2 (unbuffered) or MHB pH 5.5 media in the presence and absence of 100 μM $FeSO_4$, and the polymyxin B MIC was determined in the same medium from at least three independent determinations.

$FeSO_4$ due to iron toxicity (Fig. 3B). Complementation, genetic linkage, and DNA sequence analyses revealed that all (7 of 7) TIVAR− strains contained mutations within the PhoPQ/PmrAB regulatory pathway; and TIVAR− strains contain similar or, in some cases, identical mutations that have circulated among diseased animals and humans. These findings suggest that conversion of TIVAR− strains to the TIVAR+ phenotype may occur in high Fe^{3+} soil/water environments.

Next, we evaluated the role of iron in the susceptibility of microbes to antibiotics in MHB, the standard medium used clinically for antimicrobial susceptibility testing. While growth of TIVAR+ and TIVAR− *S.* Typhimurium strains in MHB in the presence of 100 μM $FeSO_4$ resulted in a mild increase in PMB resistance relative to MHB (2 vs. 0.5 μg/ml), growth in MHB pH 5.5 medium in the presence of 100 μM $FeSO_4$ resulted in a marked increase in PMB resistance relative to MHB pH 5.5 (64 vs. 0.5–1 μg/ml) (Fig. 3C). These levels are similar to those exhibited upon growth in LPM pH 5.5 medium. These findings suggest that drug resistance may also be induced in iron-abundant conditions that may exist within *in vivo* and *ex vivo* environs.

3.4. Bacteria Exhibit High-level Antibiotic Resistance Only in a Subset of Host Tissues

The degree of antibiotic resistance as a function of growth within cultured macrophages was evaluated using TIVAR+ and TIVAR− bacteria that have comparable virulence in a murine model of typhoid fever (Heithoff et al., 2012). TIVAR+ *Salmonella* derived from infected cultured macrophages exhibited high-level PMB resistance via MIC determination *in vitro*, whereas TIVAR− *Salmonella* did not (Fig. 4A). Further, PMB treatment of infected cultured macrophages showed that internalized TIVAR+ bacteria were much more resistant to PMB than TIVAR− bacteria ($P < 0.001$; Fig. 4B) (the inability of PMB to clear TIVAR− bacteria is presumably due to relatively low intracellular activity of cationic antimicrobial peptides (Buyck et al., 2013; Carryn et al., 2003)). These findings indicate that internalized TIVAR+ bacteria are refractory to killing by PMB relative to bacteria that do not exhibit the TIVAR phenotype.

Next, the degree of antibiotic resistance exhibited by bacteria derived from infected mice was evaluated in a murine model of typhoid fever. Mice were intraperitoneally infected with TIVAR+ or TIVAR− *Salmonella*, and bacteria derived from the tissues of septic mice were assessed for antibiotic susceptibility via MIC determination *in vitro*. TIVAR+ *Salmonella* exhibited high-level PMB resistance (MIC = 128–256 μg/ml) only when derived from a subset of host tissues, including mesenteric lymph nodes, lung, liver, spleen and gall bladder (Fig. 5). In contrast, the TIVAR phenotype was not manifested in TIVAR+ bacteria derived from other host tissues and from biological samples routinely used to detect bacterial infection (blood, feces, urine), or in TIVAR− bacteria derived from all tissues and biological samples tested. Since circulating PMB levels in treated patients and experimental rat infections range from 0.5 to 2.5 mg/l (Omri et al., 2002; Sandri et al., 2013; Zavascki et al., 2008), our findings indicate that TIVAR promotes drug resistance at certain host sites even when drug levels in circulation of mice are potentially 100 times higher than in treated patients.

3.5. Antibiotic Treatment Fails to Control Bacterial Infection and Promotes the Emergence of Drug-resistant Mutants

Polymyxin B is a last-line therapy to treat infections caused by multidrug-resistant Gram-negative bacteria in critically ill patients (Sandri et al., 2013; Zavascki et al., 2008). Thus, we evaluated whether PMB treatment was able to control TIVAR+ *Salmonella* infection in a murine model of typhoid fever. All untreated mice infected with TIVAR+ or TIVAR− bacteria died within 5 days of infection (Fig. 6A). PMB treated mice infected with TIVAR− bacteria survived at least 10 days post-infection, whereas all PMB treated mice infected with TIVAR+ bacteria died within 7 days of infection ($P < 0.001$). Since the PMB dose given (30 mg/kg/day) was 6 to 20-fold higher than that used to treat human or experimental rat infections (1.5 to 5 mg/kg/day (Abdelraouf et al., 2012; Bergen et al., 2012; Landman et al., 2008)), human infection with TIVAR+ bacteria would likely not be controlled by prescribed dosages. In contrast, ciprofloxacin (CIP) treated mice infected with TIVAR+ *Salmonella* survived at least 10 days post-infection at dosages used to treat human or experimental mouse infections (30 mg/kg/day (Fantin et al., 2009; Guillard et al., 2013)). These data are consistent with the bacterium's susceptibility to CIP in MIC testing under LPM pH 5.5 conditions (Fig. 1A), and the drug's established intracellular activity (Carryn et al., 2003).

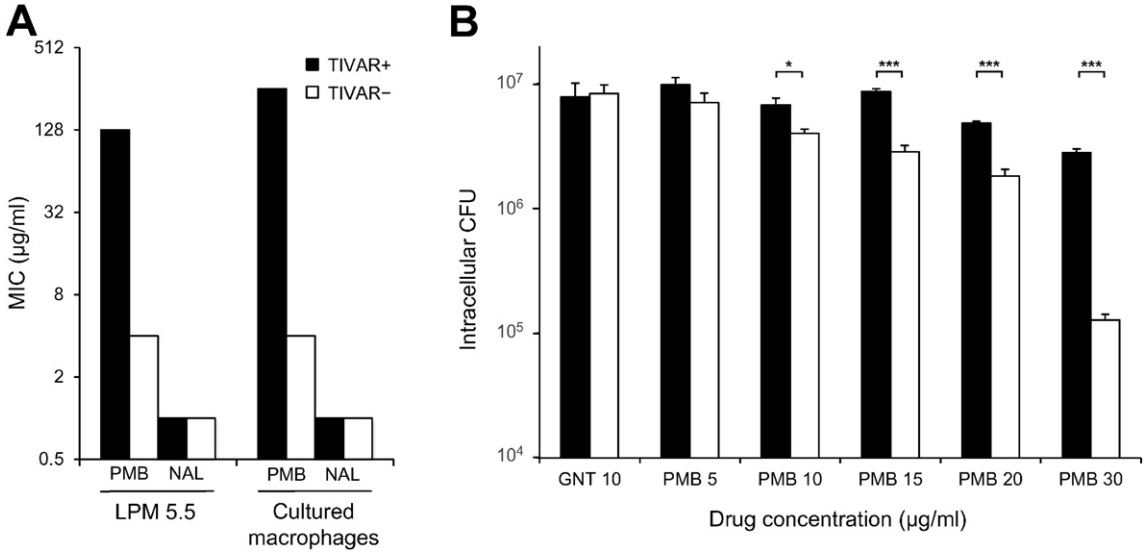

Fig. 4. *Salmonella* exhibit high-level antibiotic resistance within cultured macrophages and are refractory to killing by antibiotic treatment. (A) Antimicrobial susceptibility of TIVAR+ *S.* Typhimurium 14028 and TIVAR− *S.* Typhimurium var. 5 (04)-9639 derived from cultured RAW264.7 murine macrophages was evaluated via MIC determination on LPM pH 5.5 medium containing polymyxin B (PMB) or nalidixic acid (NAL). MIC values were the result of 2 to 3 independent determinations performed in triplicate. (B) Efficacy of antibiotic administration on the proliferation of TIVAR+ and TIVAR− *Salmonella* within cultured RAW264.7 murine macrophages (PMB, gentamicin [GNT]). Values given are the average intracellular CFU ± SEM derived from 2 to 4 independent determinations performed in triplicate. Log transformed intracellular CFU data were analyzed for statistical significance using ANOVA; $^{*}P < 0.05$, $^{***}P < 0.001$.

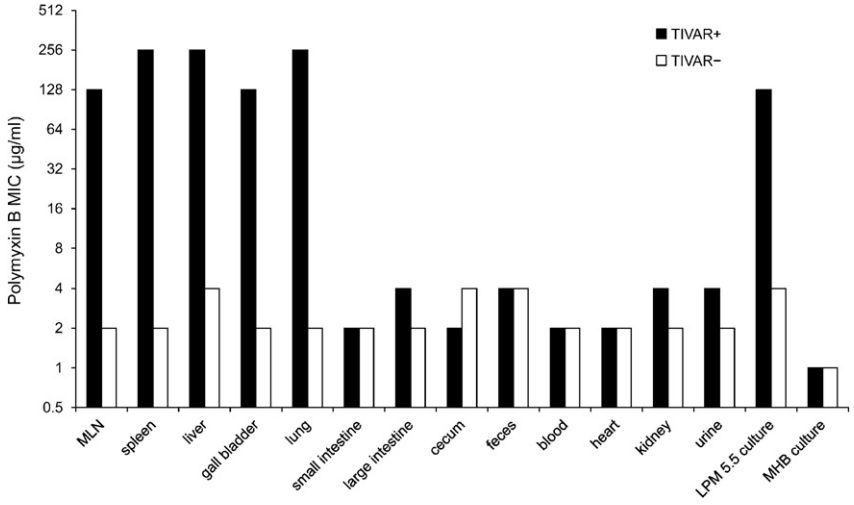

Fig. 5. *Salmonella* exhibit high-level antibiotic resistance when derived from a subset of host tissues and cells. BALB/c mice were infected with TIVAR+ *S.* Typhimurium 14028 or TIVAR− *S.* Typhimurium var. 5 (04)-9639 (i.p. dose 10^3 CFU), and bacteria were derived from host tissues of septic mice. Antimicrobial susceptibility was assessed by MIC determination under inducing conditions (LPM pH 5.5 medium). TIVAR+ *S.* Typhimurium MT2057 (kanamycin [KAN] resistant derivative of *S.* Typhimurium 14028) and TIVAR− *S.* Typhimurium var. 5 (04)-9639 (naturally resistant to chloramphenicol [CAM]) were used in these studies. MIC determinations for these strains contained KAN (40 μg/ml) and CAM (10 μg/ml), respectively, and were obtained from 3 to 13 mice per tissue/fluid. MLN, mesenteric lymph nodes.

Bacterial genetic mutagenesis occurs with increased frequency during infection of the host (Giraud et al., 2001; Martinez and Baquero, 2000; Nilsson et al., 2004). Here, we compared the frequency at which permanent PMB resistant mutants (PMB^r) arise in *Salmonella* derived from cell culture versus that from antibiotic-treated or untreated animals. TIVAR+ or TIVAR− *Salmonella* derived from overnight LB culture (*in vitro*) or from the spleens of septic mice (*in vivo*), obtained from untreated (day 5) and treated animals (TIVAR+, day 7; TIVAR−, day 10), were plated on LB medium containing PMB. Both TIVAR+ and TIVAR− bacteria derived from the spleens of untreated mice exhibited a 60-fold increase in frequency of PMB^r mutants relative to *in vitro* grown bacteria (Fig. 6B). Furthermore, TIVAR+ bacteria exhibited an additional ~5-fold increase in mutation frequency when derived from treated (day 7) versus untreated (day 5) mice ($P < 0.001$). This implies that the increased lifespan of treated mice allows for additional rounds of bacterial replication, selection, and mutagenesis, ultimately resulting in an increased frequency of permanent drug-resistant mutants (note that successful antibiotic treatment of mice infected with TIVAR− bacteria precluded recovery of PMB^r mutants from these animals (day 10)). DNA sequence analysis revealed that all PMB^r mutants derived from TIVAR+ bacteria *in vitro* (10/10) and *in vivo* (4/4) harbor mutations within *pmrAB* genes, which are known to confer PMB resistance via lipopolysaccharide (LPS)

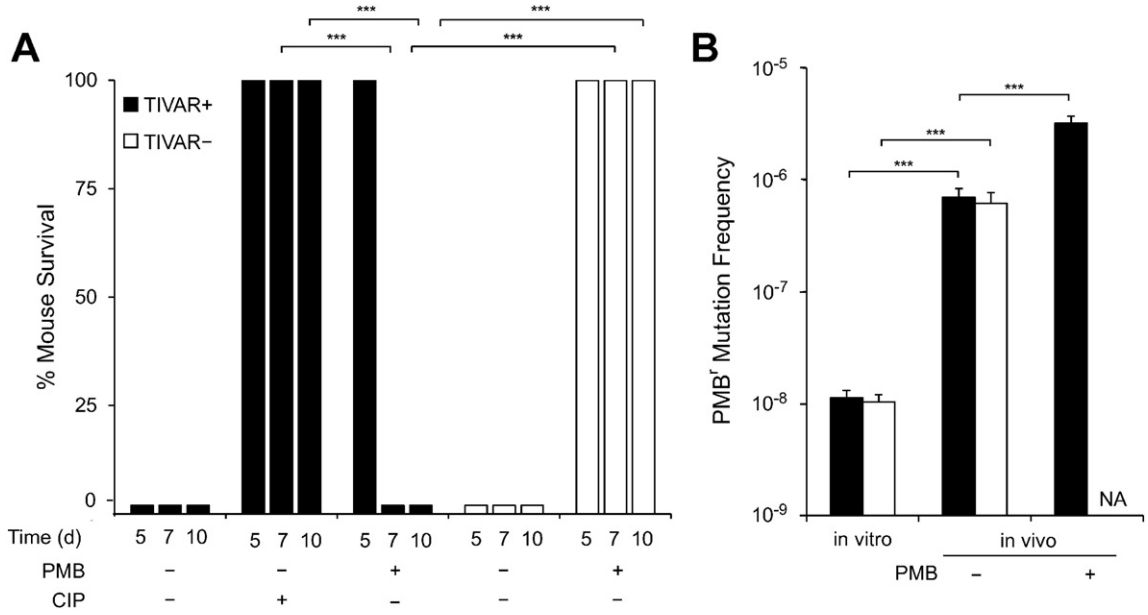

Fig. 6. Antibiotic treatment is ineffective at controlling *Salmonella* infection and promotes the emergence of drug-resistant mutants. (A) BALB/c mice infected with TIVAR+ *S.* Typhimurium 14028 or TIVAR− *S.* Typhimurium var. 5 (04)-9639 (i.p. dose 10^2 CFU) were treated (or mock-treated) with a twice-daily PMB or ciprofloxacin dosing regimen (30 mg/kg/day; i.p. route; 10 mice/cohort). Mouse survival was assessed for 10 days post-infection. Statistical significance for difference in proportions of animal survival was calculated using Chi-square; $^{***}P < 0.001$. (B) TIVAR+ or TIVAR− *Salmonella* derived from overnight LB culture (*in vitro*) or from the spleens of septic mice (*in vivo*), obtained from untreated (day 5) and treated animals (TIVAR+, day 7; TIVAR−, day 10), were plated on LB medium containing PMB (16 μg/ml). TIVAR+ *S.* Typhimurium MT2057 (kanamycin resistant derivative of *S.* Typhimurium 14028) and TIVAR− *S.* Typhimurium var. 5 (04)-9639 were used in these studies. Values given are the average mutation frequency (no. of PMB^r colonies/total no. of colonies plated) ± SEM derived from 10 independent determinations. NA (not applicable): successful antibiotic treatment of mice infected with TIVAR− bacteria precluded recovery of PMB^r mutants from these animals. Log transformed intracellular PMB^r mutation frequency data were analyzed for statistical significance using ANOVA; $^{***}P < 0.001$.

modifications that lead to membrane stabilization (Gunn, 2008; Kawasaki, 2012). Together, these findings indicate that antibiotic treatment fails to control bacterial infection caused by TIVAR + bacteria, and promotes the emergence of permanent drug-resistant mutants due to bacterial survival and growth during treatment.

4. Discussion

Since the discovery of penicillin by Alexander Fleming in 1928, antibiotic resistance has become widespread and plagues our standard of care today. We have identified a mechanism of antibiotic resistance (TIVAR) that promotes resistance *in vivo* at levels of drug that far exceed clinical doses routinely used for treatment. This mechanism has been overlooked in part because it is reversible and only operates within a subset of host tissues and cells, whereby the host milieu induces changes in the bacterium such that pathogens may become transiently resistant to high doses of certain antibiotics. TIVAR leads to treatment failure in animals, and may lead to patient treatment failure with the emergence of drug-resistant mutants due to bacterial survival and growth during antibiotic exposure. These findings call into question standardized MIC testing that has guided physician antibiotic treatment practices and drug development for the last several decades as it may not correlate with patient outcome.

Although the standard 7–10 day antibiotic treatment regimen is usually sufficient to clear microbial infections, some patients fail to respond and require prolonged therapy, higher dosing or alternative antibiotics. This is particularly confounding in cases involving immune competent individuals where bacterial ID and AST results predict drug sensitivity. Why does this occur and what are the possible implications? Although bacteria may be susceptible to antibiotics in the laboratory, certain host sites may present a unique biochemical environment that induces changes in the bacterium so they become transiently resistant to high doses of certain antibiotics (Fig. 7). This results in a large, transiently-resistant bacterial population from which permanent drug-resistant mutants may arise via standard mutational mechanisms. Supporting this hypothesis, *Salmonella* exhibited PMB resistance only when derived from a subset of host tissues (lung, liver, spleen), but not from other host tissues or from biological samples routinely used to detect bacterial infection (blood, feces, urine). PMB treatment failed to control bacterial infection in a murine model of typhoid fever and was associated with an increased frequency of permanent drug-resistant bacteria. This was presumably due to the increased lifespan of treated mice, allowing for additional rounds of bacterial replication, selection, and mutagenesis during antibiotic exposure. Additionally, due to cross resistance between polymyxins and host antimicrobials (Band and Weiss, 2014), exposure to host cationic antimicrobial peptides may provide selective pressure for drug-resistant mutants to arise as soon as a bacterial infection is established and long before antibiotic therapy is even started.

The TIVAR mechanism of resistance to cationic peptides is mediated by the PhoPQ/PmrAB regulatory system. Phenotypic switching from susceptibility to resistance was found to be more rapid than that of resistance to susceptibility, providing a means for retention of resistance during bacterial dissemination from permissive host sites (e.g., macrophages) in treated animals. Thus, macrophages may serve as potential reservoirs for persistent infections caused by intracellular bacteria. Additionally, the presence of Fe^{3+} triggered resistance in otherwise non-permissive conditions, indicating that resistance induction is not limited to the macrophage phagosome, but may also occur in iron-abundant environments that may exist *ex vivo* (soil/water) or *in vivo* (stomach) after ingestion of iron-rich foods (Wösten et al., 2000). This raises the possibility that resistance may be inadvertently triggered by diet; underlying conditions in the patient (*e.g.*, iron overload during hemochromatosis (National Institutes of Health, 2015)); or by clinical interventions that may counteract drug efficacy (*e.g.*, treatment of uncomplicated urinary tract infections to lower the pH of urine via ascorbic acid administration (Carlsson et al., 2001)).

A large proportion of antimicrobial use is directed to therapeutic and prophylactic use in livestock, which has been associated with the emergence of multidrug-resistant bacteria that have disseminated worldwide (Cloeckaert and Schwarz, 2001). As such, TIVAR has potential negative impacts on livestock production, with an increased risk of zoonotic transmission of multidrug-resistant pathogens to humans whereby therapeutic options would be even further constrained (Singer et al., 2003; Wegener, 2012). Moreover, since the induction of resistance was responsive to subtle changes in environmental conditions, management practices and environmental conditions inherent to livestock production have the potential to inadvertently trigger antibiotic resistance and the emergence of multidrug-resistant mutants; *e.g.*, diet; the use of antibiotics in feeds as growth promotants; and/or exposure to environmental variables that may induce TIVAR resistance (livestock waste, subtherapeutic concentration of antimicrobials, and/or passage through different classes of stock).

TIVAR was evident in pathogenic serotypes of *Salmonella* and *Yersinia*, and similar mechanisms of resistance may be prevalent across the microbial realm. While it is likely that evolution of TIVAR predates antibiotic use, it is likely to play into the alarming rates of emerging antibiotic resistant bacteria. Even in the most advanced hospitals, high drug doses are given to infected patients without the knowledge that

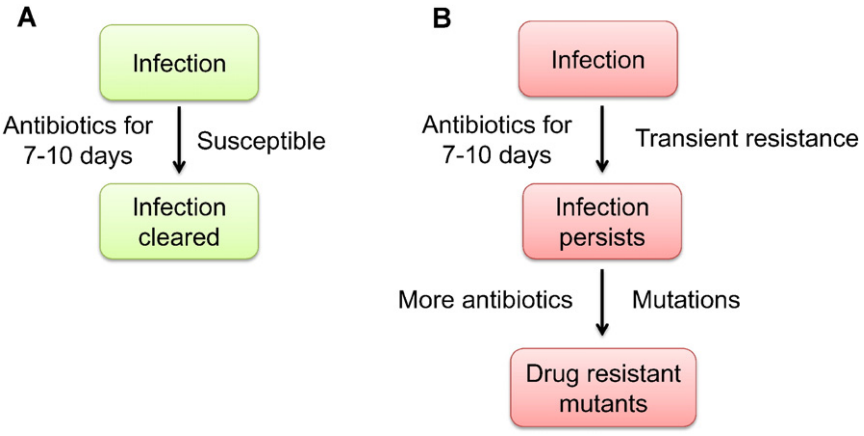

Fig. 7. TIVAR overview. (A) The standard 7–10 day antibiotic treatment regimen is sufficient to clear most bacterial infections. (B) Antibiotic treatment of persistent infections may be ineffective at controlling bacterial proliferation as certain host microenvironments may stimulate changes in the bacterium that result in the induction of transient resistance to high doses of antibiotics. This creates a major bacterial population that is transiently resistant to certain antibiotics, from which drug-resistant mutants may arise via established mutational mechanisms.

the host milieu may render bacteria inherently resistant to the antibiotics prescribed to control them. Nonetheless, for physicians managing cases where an antibiotic fails to clear an infection, rather than extending the current treatment or increasing the dose, a potentially more effective therapeutic option is prescription of another antibiotic indicated for the suspected pathogen. Unfortunately, standard *in vitro* testing methods may also inadvertently exclude antibiotics with potent efficacy, thereby unnecessarily limiting therapeutic options for multidrug-resistant pathogens as has been recently shown with azithromycin (Lin et al., 2015). Additionally, *in vitro* assessments of antibiotic resistance mutation frequency are not accurate predictors of clinical resistance (Thulin et al., 2015). Our findings support the use of host models as an important adjunct approach for antibiotic drug development and susceptibility testing, and may further help to modify current treatment guidelines to improve therapeutic intervention and diminish the emergence of multidrug-resistant pathogens.

Declaration of Interests

The authors have no competing interests to declare.

Author Contributions

Author contributions: J.Z.K., D.M.H., W.R.S, and M.J.M designed research; J.Z.K., D.M.H., W.R.S., and S.C.E. performed research; J.Z.K., D.M.H., W.R.S., S.C.E., J.K.H., J.D.M., J.W.S., and M.J.M. analyzed data; and J.W.S. and M.J.M. wrote the paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2015.08.012.

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